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A Peptide Based on the Sequence of the CDR3 of a Murine Anti-DNA mAb Is a Better Modulator of Experimental SLE Than Its Single Amino Acid-Substituted Analogs

Naama Brosh, Heidy Zinger, Mati Fridkin, and Edna Mozes[1]

Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

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A peptide based on the complementarity determining region (CDR) 3 of a pathogenic anti-DNA 16/6 Id$^+$ monoclonal antibody was previously shown to be a dominant T-cell epitope in experimental SLE, and to be capable of inhibiting SLE-associated proliferative responses. Single amino acid-substituted analogs of pCDR3 were designed and analyzed for their ability to stimulate or inhibit the proliferation of a pCDR3-specific T-cell line. Alterations in positions 9 and 10 neutralized the proliferative potential of pCDR3, whereas alterations in positions 6–8 and 11–15 retained the proliferative potential of the peptides. Similar to pCDR3, its analogs Ala11 and Nle13 inhibited efficiently the *in vivo* priming of lymph node cells either to pCDR3 or to the human monoclonal anti-DNA 16/6 Id$^+$ antibody. Substituting both positions 11 (Tyr → Ala) and 13 (Met → Nle) reduced this inhibitory capacity compared to the single substituted analogs. Also, truncation of pCDR3 at the C- and/or N-terminus obliterated the inhibitory activities of the peptide. Analogs Ala11 and Nle13 immunomodulated serological and clinical manifestations of experimental SLE. Nevertheless, the original pCDR3 was a more efficient modulator of the disease. © 2000 Academic Press

Key Words: **CDR-based peptides; altered peptide ligands; experimental SLE.**

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of autoantibodies to nuclear proteins and nucleic acids, accompanied by clinical manifestations (e.g., leukopenia, thrombocytopenia, and kidney damage (1). Induction of experimental SLE in mice has been previously reported in our laboratory, using the human (2) or murine (3) monoclonal anti-DNA antibodies that bear the common idiotype (Id) 16/6. The 16/6 Id-immunized mice produced high levels of autoantibodies, including anti-DNA and anti-nuclear protein antibodies, as well as anti-idiotypic antibodies of the 16/6 Id network. Additionally, SLE-afflicted mice developed leukopenia, proteinurla, and high intensity immune complex deposits in their kidneys (2–4). Induced experimental SLE was found to share features with the (NZB × NZW)F1 spontaneous SLE model. Thus, sequencing of the variable regions coding for the heavy and light chains of anti-DNA mAb isolated from mice afflicted with experimental SLE show high homology with the variable regions of anti-DNA mAb isolated from (NZB × NZW)F1 mice (5).

The autoantigen(s) in SLE is unknown. Nevertheless, T-cell epitopes derived from anti-DNA mAbs were characterized. These include peptides derived from anti-DNA autoantibodies as was shown in the (NZB × NZW)F1 lupus-prone mice (6, 7). To elucidate the pathogenic epitopes in the induced model of experimental SLE we have synthesized two peptides based on the sequence of the complementarity determining regions (CDR) of the pathogenic murine monoclonal anti-DNA, 16/6 Id$^+$ antibody (designated 5G12). The peptides, pCDR1 and pCDR3, were shown to induce T-cell proliferation and mild SLE upon active immunization of BALB/c and SJL mice, respectively (8). Furthermore, when administered in PBS, the peptides were able to inhibit T-cell stimulation in mice actively immunized with the same peptides, or with the whole 16/6 Id-bearing pathogenic antibodies of either murine or human origin (8). The involvement of pCDR3 in the pathogenicity of SLE was further supported by showing that syngeneic immunization with a T-cell line specific to pCDR3 induced mild experimental SLE in SJL mice (9). Also, naive splenocytes of (NZB × NZW)F1 lupus-prone mice were shown to proliferate in response to pCDR3 (10).

SLE, as other autoimmune diseases, represents an inappropriate immune attack on self-tissue. Current

[1] To whom correspondence should be addressed at Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel. Fax: +972 8-9344141. E-mail: edna.mozes@weizmann.ac.il.

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therapies for almost all human autoimmune diseases utilize potent and nonspecific immnosuppressive agents. Another approach to treatment of autoimmunity is the use of immunodominant or altered peptide ligands (APLs) with the objective of reestablishing the immunological tolerance in an antigen-specific manner.

Peptide analogs with substitutions at amino acids contacting the TCR (11) have the capacity to modulate T-cell responsiveness and as such hold promise for antigen specific immunotherapy of T-cell-mediated autoimmune pathology (12, 13). Certain APLs have been shown to act as antagonists of T-cell activation in response to wild-type peptide antigens *in vitro* (12, 14). This ability has also been harnessed to show inhibition of autoimmune diseases *in vivo* following simultaneous immunization with wild-type peptide and the antagonist APL (15–18).

In this report we have designed a series of one amino acid-substituted analogs of pCDR3 and analyzed their ability to stimulate the proliferative responses of a T-cell line specific to pCDR3. Additionally, we have compared the ability of pCDR3 and its analogs to inhibit *in vitro* proliferative responses of the pCDR3-specific T cell line and also the *in vivo* priming of lymph node cells either by pCDR3 or by the whole human monoclonal anti-DNA 16/6 Id Ab. Finally we tested the ability of pCDR3 and selected analogs to immunomodulate experimental SLE induced by the human 16/6 Id mAb.

MATERIALS AND METHODS

Animals

SJL inbred female mice (Jackson Laboratory, Bar Harbor, ME) were used at the age of 8–10 weeks.

Synthetic Peptides and Peptide Analogs

The peptides used were pCDR3, which is based on the CDR3 of the murine anti-DNA monoclonal antibody 5G12 (2), reversed pCDR3, and pCDR3-based analogs. Peptide amino acid sequences are detailed in Table 2. Peptides were prepared using an automated multiple peptide synthesizer (Model AMS 422; Ambimed Langenfeld, Germany) using the company protocols *N*-α-butyloxycarbonyl (*t*-BOC) strategy (19).

Monoclonal Antibody

The human 16/6 Id anti-DNA mAb (IgG1/k) was secreted by hybridoma cells grown in culture and the antibody was purified on a protein G-Sepharose column (Pharmacia, Sweden) according to the manufacturer's instructions.

Immunizations

Mice were injected intradermally into the hind footpads with the immunizing peptide (20 μg) or the 16/6 Id mAb (2 μg), emulsified in complete Freund's adjuvant (CFA) in a total volume of 100 μl. For experimental SLE induction, mice were immunized with the 16/6 Id mAb, as detailed above. After 3 weeks the animals were given a booster injection of the 16/6 Id mAb (2 μg) in PBS in a total volume of 100 μl.

Establishment of pCDR3-Specific T-Cell Lines

SJL-derived T-cell lines specific to peptide pCDR3 were established from popliteal LN cells of immunized mice (as detailed above), according to previously published methods (20). Cells were exposed to the stimulating peptide (15 μg/ml) presented by irradiated (3000 rad) syngeneic spleen cells every 14 days.

Proliferative Response of a pCDR3-Specific T-Cell Line

Seven or 10 days after antigenic stimulation, cells of the T-cell line were tested for their proliferative responses. Cells (10^5/well) were cultured with 0.5×10^6 irradiated syngeneic spleen cells in the presence of different concentrations of the peptides. Cultures were established in flat-bottom microtiter plates in 200 μl enriched medium containing 10% FCS. At the end of a 48-h incubation period, 0.5 μCi of [^{3}H]thymidine (5 Ci/mmol, Nuclear Research Center, Negev, Israel) was added. Sixteen hours later, cells were harvested and radioactivity was counted by a β counter (20). Results were expressed as cpm or stimulation indices (SI) that were calculated as follows: the cpm of peptide-stimulated triplicate wells divided by the CPM of cells incubated with medium only.

Inhibitory Capacity of the pCDR3-Based Analogs on the pCDR3-Specific T-Cell Line

Seven or 10 days after antigenic stimulation, cells of the T-cell line (10^4/well) were cultured with 0.5×10^6 irradiated syngeneic spleen cells in the presence of pCDR3 (50 μg/ml) and of the different analogs (500 μg/ml). Cultures were established in flat-bottom microtiter plates in 200 μl enriched medium containing 10% FCS. At the end of a 48-h incubation period, 0.5 μCi of [^{3}H]thymidine (5 Ci/mmol, Nuclear Research Center, Negev, Israel) was added. Sixteen hours later, cells were harvested and radioactivity was counted by a β counter (20). Results were expressed as percentage (%) inhibition that was calculated as follows: % Inhibition = $(1-(\Delta\text{cpm}\ (A-B)/\Delta\text{cpm}\ (C-D))) \times 100$, where A = mean triplicate of peptide-stimulated wells of the treated animal group, B = mean triplicate of medium-stimulated wells of the treated animal group, C = mean triplicate of peptide-stimulated wells of the untreated animal group; and D = mean triplicate of medium-stimulated wells of the untreated animal group.

Proliferative Response of Lymph Node-Derived T Cells

Eleven days after immunization with pCDR3 or with the 16/6 Id mAb, lymph nodes (LN) were harvested and pooled. Cells (10^6/well) were cultured in the presence of different concentrations of the immunizing agent. Cultures were established in 200 μl enriched medium containing 1% syngeneic NMS in flat-bottom microtiter plates. At the end of a 4-day incubation period, 0.5 μCi of [^{3}H]thymidine (5 Ci/mmol; Nuclear Research Center, Negev, Israel) was added to the cultures. Sixteen hours later, cells were harvested and radioactivity was counted by a β counter. Results are expressed as percentage (%) inhibition that was calculated as described above.

Anti-DNA Antibody Detection Assay

For the detection of specific antibodies in sera of immunized mice, ELISA was carried out as described before (3). Briefly, flat bottom maxi-sorb plates (Nunc, Roskilde, Denmark) were coated with 10 μg/ml methylated BSA (Sigma). The plates were washed and coated with 10 μg/ml denatured calf thymus DNA (Sigma). After incubation with the antigen, plates were washed and blocked for overnight with 1% ovalbumin (Sigma) both in PBS. Diluted sera were then added for 2 h, followed by incubation for 1 h, with 50 μl of 0.8 μg/ml of goat anti-mouse IgG (gamma chain Fc specific; Jackson Immuno Research, West Grove, PA).

Detection of Clinical and Pathological SLE-Associated Manifestations

Proteinuria was measured by a semiquantitative method, using a Combistix kit (Ames Division, Bayer Diagnostics, Slough UK). Levels of proteinuria were calculated according to the manufacturer's scale where +1 equals 0.3 g/L, +2 equals 1 g/L, +3 equals 3 g/L. For immunohistology, frozen kidney cryostat sections (6 μm thick) were fixed and stained with FITC-conjugated goat antibodies to mouse IgG, γ-chain specific (Jackson Immunoresearch).

RESULTS

Design and Synthesis of pCDR3-Based Analogs

Based on the amino acid sequence of the pCDR3 peptide, 17 analogs with a single amino acid substitution were designed and synthesized (Table 1). The amino acid positions chosen for substitution were selected according to the putative attachment points of the peptide in the groove of the TCR/MHC class II complex (21). The substituting amino acids were chosen following principles for the evaluation of key functional amino acids in biologically active peptides. For example, several positions were substituted by the small nonpolar alanine (i.e., alanine screen) (22). Substitutions of phenylalanine at position 6 by glycine and threonine, in addition to alanine, were intended to further evaluate the importance of aromaticity and the possible effect of a polar-nonionazable moiety (OH function) on activity. Substitution of tryptophan at position 8 by leucine was intended to evaluate the importance of hydrophobicity (aliphatic rather than aromatic) on activity. Substitution of glutamic acid at position 9 by lysine was intended to evaluate the effect of charge reversal on the activity, while the insertion of glycine was aimed at using an alanine-like, small, nonpolar, nonhydrophobic and a potential structural-bender, substitution. Substitution of aspartic acid at position 14 by lysine was intended to evaluate potential results of charge reversal while introduction of serine at the same position was aimed at the evaluation of a possible effect of a polar noncharge residue capable of forming internal hydrogen bonding. Substitution of methionine at position 13 by norleucine was based on a well-established information that this isosteric modification in peptides led, almost as a rule, to derivatives without major or with little reduction of activity (23). Further, the substitution of the oxidative-labile methionine by the stable residue norleucine has a marked effect on the peptide's stability and thus reproducibility of assays.

TABLE 1
Amino Acid Substitutions of pCDR3

Amino acid sequence of pCDR3:
Y_1-Y_2-C_3-A_4-R_5-F_6-L_7-W_8-E_9-P_{10}-Y_{11}-A_{12}-M_{13}-D_{14}-Y_{15}-W_{16}-G_{17}-Q_{18}-G_{19}-S_{20}

Position	Amino acid substitution		
5	Ala		
6	Ala	Gly	Thr
7	Ala		
8	Ala	Leu	
9	Ala	Lys	Gly
10	Ala		
11	Ala		
13	Ala	Nle*	
14	Lys	Ser	
15	Ala		

* Nle, norleucine.

Effect of pCDR3-Based Analogs on a pCDR3-Specific T-Cell Line

For the initial selection of active analogs we used a T-cell line specific for pCDR3 (9). As can be seen in Fig. 1A, some of the analogs (Ala6, Thr6, Ala7, Leu8, Ser14) induced proliferation of a pCDR3-specific T-cell line in rates comparable to those induced by wild-type pCDR3 (which induced proliferation of 100-fold over the background). Analysis of inhibition assays of pCDR3-specific proliferation by the analogs revealed that several

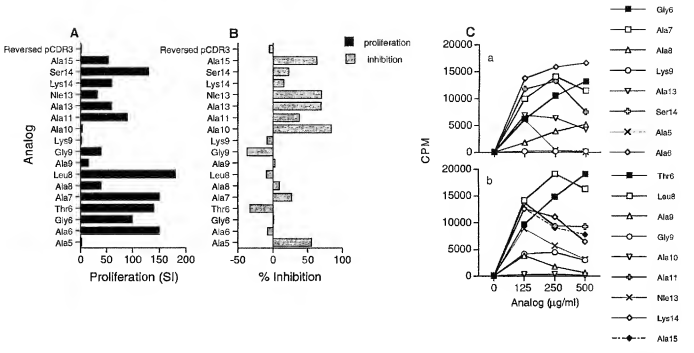

FIG. 1. Proliferation and inhibition of a pCDR3-specific T-cell line by pCDR3-based analogs. Cells of the pCDR3-specific T-cell line (5×10^4) were incubated in triplicate wells for 48 h with syngeneic (SJL) irradiated (3000 rad) splenocytes as APCs, and with the different pCDR3-based analogs at 5–100 μg/ml. Representative results are shown for 50 μg/ml (A). Proliferation results are represented as stimulation index (SI) where the background (cells + medium) levels of proliferation were 79 ± 8 cpm for the first plate, 88 ± 10 cpm for the second plate, and 165 ± 15 cpm for the third plate. SI for wild-type pCDR3 was 100-fold over the control. For the analog competition assay (B), the cells were incubated as described above with 50 μg/ml pCDR3 and 125, 250, and 500 μg/ml of the different analogs. The competition results are represented as percentage (%) inhibition only for 500 μg/ml. For a dose response proliferation for the different analogs (C [a,b]) the cells were incubated as described above with 125, 250, and 500 μg/ml of the different analogs and the results are represented as counts per minute ± standard deviation (cpm ± SD). The results of are representative of three experiments.

analogs (Ala5, Ala10, Ala13, Nle13, and Ala15) were efficient inhibitors of the proliferative capacity of the pCDR3-specific T-cell line (Fig. 1B). As can be seen, the control peptide, reversed pCDR3, did not induce proliferation (Fig. 1A) or inhibition (Fig. 1B), under the same experimental conditions. To rule out the possibility that the inhibitory effects may not be specific but due to "toxicity," we have added the same concentrations of analogs (125, 250, or 500 μg/ml) to wells containing concanavalin A and T cells. None of the analogs reduced concanavalin A-induced proliferation of the T cells (data not shown). Also, a dose-response proliferation assay for individual analogs excluded the possibility of toxicity (Fig. 1C). These results were reproducible with several pCDR3-specific T-cell lines. Analogs that inhibited more than 50% of the proliferative response of the pCDR3-specific T-cell line (Ala 5, Ala10, Ala11, Ala13, Nle13, and Ala15) were chosen for further analysis as putative modulators of experimental SLE.

Effects of pCDR3-Based Analogs on the in vitro Recall Response of pCDR3 and 16/6 Id mAb-Immunized Mice

The chosen analogs were analyzed as *in vivo* inhibitors of T-cell priming induced by pCDR3, and by the human 16/6 Id mAb. The human 16/6 Id mAb was chosen here as a multideterminant anti-DNA mAb that was previously shown to induce a lupus-like disease in naive mouse strains (2). The analysis focused on pCDR3-based analogs that could show consistent inhibition of proliferation of the pCDR3-specific T-cell line over several experiments. The analogs Ala5, Ala10, and Ala15 did not confer reproducible inhibition and were not used further. Thus, we continued to analyze analogs Ala11, Ala13, and Nle13. Figure 2 shows a representative experiments examining the ability of the analogs to inhibit the *in vivo* priming to pCDR3 and to the whole anti-DNA autoantibody 16/6 Id. All three analogs inhibited the proliferation of LN-derived T cells to the immunizing peptide pCDR3. Analogs Ala11 and Ala13 resembled pCDR3 in their activity, while Nle13 inhibited pCDR3-stimulated T cells to a higher extent. Reversed pCDR3 used as control did not effect pCDR3-stimulated proliferation (Fig. 2A). When animals were immunized with the 16/6 Id mAb, again, all three analogs inhibited 16/6 Id-induced T-cell proliferation. In this case, the analog Ala13 showed a somewhat higher inhibitory activity (Fig. 2B). Thus, it appeared that positions 11 and 13 of the pCDR3 peptide are of importance for the design of a negative-

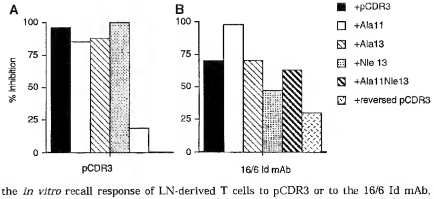

FIG. 2. Inhibition of the *in vitro* recall response of LN-derived T cells to pCDR3 or to the 16/6 Id mAb, by pCDR3-based analogs. Twenty-six SJL mice were immunized with 20 μg/animal of pCDR3 (A) or with 2 μg/animal of the 16/6 Id mAb (B), in CFA intradermally, in a total volume of 100 μl. Concomitantly, the animals were injected with 250 μg of one of the peptides: pCDR3, analog Ala11, analog Ala13, analog Nle13, with the combined analog Ala11Nle13, or with reversed pCDR3 peptide (as control), in PBS, iv, in a total volume of 300 μl. Eleven days postimmunization popliteal LN were harvested and cells (10^5/well) were incubated in triplicate wells with different concentrations of either pCDR3 (5–100 μg/ml) or with the 16/6 Id mAb (0.5–50 μg/ml). Optimal proliferation values were calculated for pCDR3 (25 μg/ml) and for the 16/6 Id mAb (50 μg/ml) and are presented as percentage (%) inhibition. Background levels (cells+medium) were 5770±514 cpm. The results are representative of three experiments.

regulating peptide analog for SLE-related T-cell responses.

In view of these results, we designed and synthesized an additional analog with two substitutions, combining analogs Ala11 and Nle13 (Table 2). The combined analog (Ala11Nle13) failed to inhibit proliferation of LN-derived T cells from pCDR3-immunized mice, as compared to the single-substituted analogs Ala11 and Nle13, and to pCDR3 itself (Fig. 2A). The combined analog Ala11Nle13 was more effective in inhibiting proliferation to the 16/6 Id mAb, but the inhibition rates were not better than those of pCDR3 or than those obtained by the single-substituted analogs (Fig. 2B). In fact, analog Ala11 demonstrated in these experiments the highest ability to inhibit the proliferation of 16/6 Id-stimulated T cells. Therefore, the combined analog had no advantage over the single-substituted analogs and was not used further.

Next, we have questioned whether pCDR3 could be truncated in its N- or C-terminus and still retain the proliferation-inhibiting activity. Therefore, three peptides were synthesized: pCDR3 truncated by two amino acids at its N-terminus, pCDR3 truncated by two amino acids at its C-terminus, and pCDR3 truncated by two amino acids both at its N- and C-termini (Table 2). These peptides were analyzed for their ability to inhibit the proliferation of LN-derived T cells stimulated by pCDR3 or by the 16/6 Id mAb. As can be seen in Fig. 3A, the N-terminus- and the C-terminus-truncated peptides lost completely their ability to induce proliferation arrest. Surprisingly, pCDR3 truncated by two amino acids at both ends retained some (about 50%) of the ability of pCDR3 to inhibit proliferation of pCDR3-immunized LN cells. When the animals were immunized with the 16/6 Id mAb and treated *in vivo* with the truncated peptides, none of the three truncated peptides could inhibit the T-cell proliferation as efficiently as pCDR3 itself (Fig. 3B). As can be seen, the C-terminus-truncated pCDR3 retained some (about 39%) of the activity. Therefore, it appears that amino acids at the N- and C-termini of the pCDR3 peptide are essential to inhibit both pCDR3- and 16/6 Id-induced proliferation.

pCDR3-Based Analogs Ala11 and Nle13 Induce Mild Experimental SLE

Active immunization (with CFA) with pCDR3 was previously shown to induce mild SLE in naive SJL mice (8). Therefore, we assessed first the ability of the

TABLE 2
Amino Acid Sequences of Peptides

Peptide	Amino acid sequence
pCDR3	Y-Y-C-A-R-F-L-W-E-P-Y-A-M-D-Y-W-G-Q-G-S
Combined analog (Ala11Nle13)	Y Y-C-A-R-F-L-W-E-P-A-A-X-D-Y-W-G-Q-G-S
N-terminal-truncated pCDR3	C-A-R-F-L-W-E-P-Y-A-M-D-Y-W-G-Q-G-S
C-terminal-truncated pCDR3	Y-Y-C-A-R-F-L-W-E-P-Y-A-M-D-Y-W-G-Q
N- and C-terminal-truncated pCDR3	C-A-R-F-L-W-E-P-Y-A-M-D-Y-W-G-Q
Reversed pCDR3	S-G-Q-G-W-Y-D-M-A-Y-P-E-W-L-F-R-A-C-Y-Y

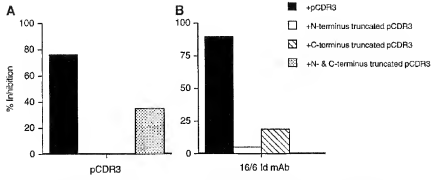

FIG. 3. Inhibition of the *in vitro* recall response of LN-derived T cells to pCDR3 or to the 16/6 Id mAb by the truncated pCDR3 peptides. Sixteen SJL mice were immunized with 20 μg/animal of the pCDR3 (A) or with 2 μg/animal of the 16/6 Id mAb (B) in CFA intradermally, in a total volume of 100 μl. Concomitantly, the animals were injected with the N-terminal-truncated pCDR3, with the C-terminal-truncated pCDR3, or with the N- and C-terminal-truncated pCDR3. Peptides pCDR3 and reversed pCDR3 were used as positive and negative controls, respectively. All peptides were injected iv, in PBS, 250 μg/animal, in a total volume of 300 μl. Eleven days postimmunization popliteal lymph nodes were harvested and cells (10^5/well) were incubated in triplicate wells with either pCDR3 (5–100 μg/ml) or with the 16/6 Id mAb (0.5–50 μg/ml). Optimal proliferation values were calculated for pCDR3 (at 50 μg/ml) and for the 16/6 Id mAb (at 50 μg/ml) and are presented as percentage (%) inhibition. Background levels (cells + medium) were 2089 ± 142 CPM. The results are representative of three experiments.

analogs to induce experimental lupus. As can be seen in Fig. 4, pCDR3 and its analogs induced different levels of anti-DNA Abs but with insignificant variance. The mice were also followed for other disease manifestations. Of the three peptides, pCDR3 induced low levels of proteinuria while urinary protein levels induced by analogs Ala11 and Nle13 were close to these measured in normal age-matched SJL mice (results not shown). At the end of the experiment the presence of immune complex deposits was assessed in the kidneys of the immunized animals. Immunization with the three peptides induced immune complex deposits in part of the mice (Table 3). Thus, pCDR3 and its two analogs were not significantly different in their ability to induce a mild lupus-like disease in SJL mice.

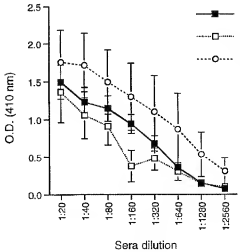

FIG. 4. Anti-DNA antibody levels in sera of mice immunized with pCDR3 and analogs Ala11 and Nle13. Individual sera of five pCDR3-immunized mice (■), five Ala11-immunized mice (□), and five Nle13-immunized mice (○) were taken 4 months after the booster injection. The sera were tested for anti-DNA antibody levels by ELISA. Results are expressed as mean OD ± SD of each group. The results are representative of two experiments.

pCDR3-Based Analogs Ala11 and Nle13 Prevent 16/6 Id-Induced Experimental SLE

We assessed the ability of pCDR3 and its analogs to prevent experimental SLE induced by immunization with the human 16/6 Id mAb *in vivo*. This experiment was designed based on a previous one, where pCDR3 and its analog Nle13 were shown to prevent pCDR3-induced disease (results not shown). Administration of analogs Ala11 and Nle13, twice in aqueous solution intravenously, concomitant with immunization with the 16/6 Id mAb in adjuvant, reduced levels of anti-DNA autoAbs, bringing them close to baseline levels

TABLE 3
Experimental SLE Induced by pCDR3 and Its Analogs Ala11 and Nle13

Immunization	Proteinuria (g/L)	Immune complex deposit intensity in kidneys		
		−	+	++
pCDR3	0.44 ± 0.19	1/5	3/5	1/5
Ala11	0.21 ± 0.082	—	1/5	4/5
Nle13	0.3 ± 0	3/5	1/5	1/5

Note. Mean values for urinary protein (±SD) were derived based on the numeric values specified by the manufacturer of the diagnostic kit (see under Materials and Methods). Mean proteinuria value of normal SJL age-matched mice is 0.16 ± 0.07. Immune complex deposit density: (−) no immune complexes, (+) moderate intensity immune complex deposits; (++) high intensity immune complex deposits. Results are representative of two experiments.

TABLE 4
Prevention of Experimental SLE by pCDR3 and Its Analogs Ala11 and Nle13

Treatment	Proteinuria (g/L)	Immune complex deposit intensity in kidneys			Percentage of animals with immune complex deposits in kidneys
		−	+	++	
16/6 immunized	0.86 ± 0.19	—	2/6	4/6	100
16/6 immunized + pCDR3	0.47 ± 0.29	5/6	—	1/6[a]	16.6
16/6 immunized + Ala11	0.52 ± 0.45	3/6	2/6	1/6[b]	50
16/6 immunized + Nle13	0.20 ± 0.15	3/6	2/6	1/6[c]	50
Normal	0.37 ± 0.16	4/5	—	1/5	20

Note. Mean values for urinary protein (±SD) were derived based on the numeric values specified by the manufacturer of the diagnostic kit (see under Materials and Methods). Immune complex deposit density: (−) no immune complexes, (+) moderate intensity immune complex deposits, (++) high intensity immune complex deposits. The data were analyzed by the Mann-Whitney test. [a]$P = 0.026$, [b]$P = 0.065$, [c]$P = 0.065$.

detected in normal age-matched SJL mice (Fig. 5). As can be seen in the figure, kinetic measurements of anti-DNA Ab levels taken 2, 3, and 4 months after end of treatment demonstrate that as a whole, pCDR3 and its analogs do not significantly differ in their ability to reduce pathogenic anti-DNA Abs. Similarly, secreted urinary protein in 16/6 Id-immunized mice was reduced by pCDR3 and by the two analogs (Table 4). Kidney damage in 16/6 Id-immunized mice was assessed by evaluation of immune complex deposit within these organs. Kidney damage is considered a most important parameter for analysis of SLE pathogenesis. Although analogs Ala11 and Nle13 reduced partially the number of animals with immune complex deposits (50%) and their intensity (Table 4), only the wild-type pCDR3 prevented significantly (down to 16.6%,) the number of animals that developed immune complex deposits and their intensity ($P = 0.026$, Table 4). This relative superiority of wild-type pCDR3 was demonstrated also in preventing pCDR3-induced SLE and in treating SJL mice, where pCDR3 and analog Nle13 were administered to animals with developed 16/6 Id-induced experimental SLE (results not shown). Figure 6 represents immunohistology of kidney sections of mice immunized with the 16/6 Id and either not treated (Fig. 6A) or treated with pCDR3 (Fig. 6C), or its analog Nle13 (Fig. 6D). The figure demonstrates the significant reduction in immune complex deposits in kidneys of the pCDR3-treated mice.

DISCUSSION

In the present report we compared and analyzed the ability of analogs of a peptidic T-cell epitope to inhibit SLE-associated T-cell responses as well as clinical manifestations in mice inflicted with experimental SLE. The original peptide was based on the CDR3 of an anti-DNA mAb derived from mice with experimental SLE, and was previously shown to induce mild SLE in naive mice by active immunization, and also to modulate SLE-associated T-cell responses when administered in aqueous solution (8). We report here that 2 of the initial 17 analogs tested were capable of inhibiting the *in vitro* recall T-cell responses stimulated by either pCDR3 or by the human anti-DNA 16/6 Id mAb. Also, the analogs were able to modulate 16/6 Id-induced experimental SLE. Nonetheless, we show here that com-

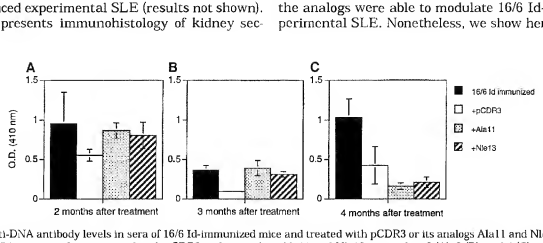

FIG. 5. Anti-DNA antibody levels in sera of 16/6 Id-immunized mice and treated with pCDR3 or its analogs Ala11 and Nle13. Individual sera of 30 16/6 Id-immunized mice treated with pCDR3 and its analogs Ala11 and Nle13 were taken 2 (A), 3 (B), and 4 (C) months after the treatment. The sera were tested for anti-DNA antibody levels in several dilutions (1:20–1:2560) by ELISA. Results were calculated for a representative sera dilution of 1:320 and were expressed as mean OD ± SE of each group. The results are representative of two experiments.

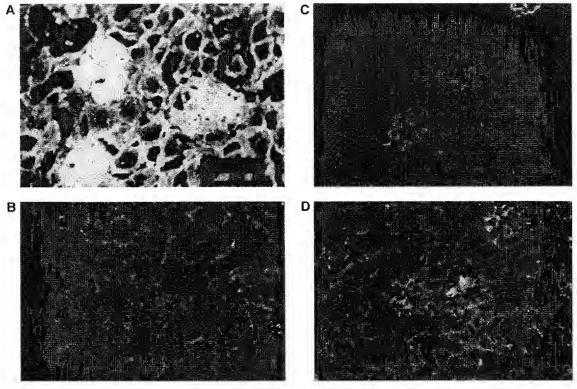

FIG. 6. Immunohistology of kidney sections from mice immunized with the 16/6 Id mAb and treated with pCDR3 or its analog Nle13. Five months after the end of the treatment, mice were sacrificed, and their kidneys were removed and analyzed for the presence of immune complex deposits as described under Materials and Methods (×400). The pictures represent samples of the different groups: 16/6 immunized mice (A), normal age-matched SJL mice (B), pCDR3-treated mice (C), analog Nle13-treated mice (D).

pared to the analogs, the original pCDR3 was a more efficient immunomodulator of experimental SLE.

Studies analyzing T-cell activation (24, 25) show that only three to four amino acid side chains of a peptide are accessible to the TCR (26, 27). These residues are referred to as primary and secondary TCR contacts (11, 28, 29). The pCDR3-based analogs were designed according to the amino acid pattern present in many T-cell epitopes (21) in order to alter the putative TCR contact residues and the resulting signals received by the TCR (30). The substituted positions spanned the putative T-cell epitope of pCDR3 (positions 6–15).

Initial analysis of the pCDR3-based analogs was carried out using stimulation and inhibition of proliferation assays of the pCDR3-specific T-cell line. The results of these assays revealed that pCDR3-based analogs belonged roughly to three major groups. Thus, analogs altered in positions 6–8 induced proliferation of the T-cell line, analogs substituted in positions 9 and 10 induced no proliferation, while analogs substituted at positions 5, 11–15 induced proliferation but also inhibited pCDR3-specific proliferative responses of the T-cell line. Alterations in positions 9 and 10 neutralized the proliferative potential of pCDR3 and these positions may be critical for activation of pCDR3-reactive T cells in SJL mice. Accordingly, positions 6–8 and 11–15 seem more permissive to alterations, as the analogs substituted at these residues retained the proliferative potential of the peptide. Analogs resulting from substitutions at positions 11–15 were capable of both triggering the proliferation of the pCDR3-specific T-cell line and inhibiting the pCDR3-specific response of this line, thus the analogs may be classified as mixed agonists/antagonists (14).

The analogs that exhibited inhibitory potential were further analyzed for their ability to inhibit the *in vivo* priming of T cells with the original pCDR3 peptide or the SLE-inducing human antibody bearing the 16/6 Id. Three analogs that were selected, Ala11, Ala13, and Nle13, presented reproducible inhibitory activity over several experiments when delivered in a tolerogenic form (i.v. in PBS). Both pCDR3 and its analogs inhibited the *in vivo* priming of SJL mice with pCDR3 or with the 16/6 Id mAb by 60–100% with insignificant differences between them. This inhibition was specific, as the reversed pCDR3 control peptide inhibited marginally the *in vivo* priming by the same antigens (Fig. 2).

A double substitution of amino acids at positions 11 and 13 was not favorable, as the combined analog (Ala11Nle13) lost partially its ability to inhibit the *in vivo* priming by pCDR3 and by the 16/6 Id antibody. It is probable that the existence of two substitutions in

the combined analog changed the fitting into the TCR/MHC class II groove, thus resulting in only partial activation of the relevant T-cell clones. In view of this, we attempted to alter the original pCDR3 by truncations. The analysis showed that of the three truncated peptides only pCDR3 truncated at both ends retained about 35% of the inhibiting activity on the *in vitro* recall T-cell response to immunization with pCDR3. Reduction of the biological activity upon N- or C-terminus truncations of immunomodulatory peptides was already reported in several systems. Thus, truncation of myasthenogenic epitopes reduced their ability to induce proliferation of specific T-cell lines (20). In another system, truncation of macrophage inflammatory protein 1 beta (MIP-1 beta) reduced its ability to dimerize and thus block viral infection (31). As for immunization with the 16/6 Id mAb, only the full-length pCDR3 acted as a negative regulator of T-cell proliferation to the 16/6 Id mAb. It is possible that truncation of pCDR3 at either end reduced its ability to fit into the TCR/MHC class II groove, and therefore the truncated peptides were unable to compete out the immunizing agent. When immunizing with pCDR3, the peptide truncated at both termini could probably fit into the groove, thus exerting some of the inhibiting activity. The activity of analogs and truncated peptides may differ depending on the immunizing agent (pCDR3 or the 16/6 Id mAb). Immunization with the whole 16/6 Id mAb probably promoted additional sets of T-cell clones and none of the truncated pCDR3 peptides could compete with the immunizing antibody and inhibit the T-cell responses.

Immunization of naive animals with pCDR3 or with the analogs Ala11 and Nle13 showed that the analogs tested had no advantage over pCDR3 as all three peptides induced mild SLE upon active immunization in adjuvant (CFA). Indeed, peptides with a therapeutic potential may present some pathogenic features. Thus, in another system, Singh and co-workers reported that intravenous injections, in aqueous solution, of peptides derived from the VH region of anti-DNA autoantibodies to young (NZB × NZW)F1 substantially delayed development of anti-DNA antibodies and nephritis and prolonged the animals' survival (7). One of these peptides (delivered in IFA) increased clinical anti-DNA Ab levels in neonatal (NZB × NZW)F1 mice, thus augmenting the disease (32). We suggest that the route of administration of lupus-associated T-cell epitopes, like pCDR3 (and its analogs), determines whether they will be "pathogenic" or "tolerogenic."

Analogs Ala11 and Nle13 modulated experimental SLE when administered in aqueous solution, concomitant with the immunizing mAb, 16/6 Id, by reducing anti-DNA Ab levels, proteinuria, and immune complex deposit in the kidneys. Yet, only pCDR3 reduced significantly the number of animals carrying immune complex deposits and their intensity in the SLE-afflicted mice. Similarly, pCDR3 modulated better (compared to the analogs) the clinical manifestations of an already developed SLE disease by reducing proteinuria and the accumulation of immune complex deposits in the kidneys (N. Brosh unpublished results). Moreover, pCDR3 could prevent experimental lupus in (NZB × NZW)F1 mice whereas analog Nle13 manifested only a mild immunomodulatory capacity in this system (33).

Several recent papers reported the successful usage of peptides based on amino acid sequences of SLE-related autoantigens in spontaneous murine lupus models. Injection of a peptide corresponding to the VH CDR3 region of a natural polyreactive autoantibody to young preautoimmune (NZB × NZW)F1 mice induced delayed development of proteinuria and 50% survival rate, but induced also DNA-reactive Abs (34). In the (SWR × NZB)F1 mouse model of lupus, a brief therapy course with peptides derived from nucleosome core histones, administered iv to 3-month-old prenephretic mice already producing pathogenic autoantibodies, markedly delayed the onset of severe lupus nephritis. Chronic therapy with the peptides injected into 18-month-old (SWR × NZB)F1 mice with established glomerulonephritis prolonged survival and halted the progression of renal disease (35). In another system, peptides that react with a pathogenic mouse monoclonal anti-DNA antibody that deposits in glomeruli were administered in a soluble form and protected mice from renal deposition of the antibody, *in vivo* (36).

Collectively, the data presented here show that the usage of the immunodominant T-cell epitope pCDR3 and its analogs Ala11 and Nle13 affected beneficially the pathogenesis of experimental SLE. Also, treatment with the original pCDR3 was shown to be advantageous as compared to the two analogs of this peptide. Additional experiments suggest that pCDR3 may serve as a key epitope in murine SLE. Thus, a T-cell line specific to pCDR3 induced experimental lupus in naive mice (9). Naive T cells from young pre-autoimmune (NZB × NZW)F1 mice responded to pCDR3 by proliferation (10). Further, pCDR3 could modulate murine lupus in (NZB × NZW)F1 both in early stages (33) or in an already developed disease (H. Zinger, E. Eilat, and E. Mozes, unpublished results). Similar results were obtained in MRL lpr/lpr lupus-prone mice (E. Eilat, H. Zinger, and E. Mozes, unpublished results).

Based on these data, we conclude that compared to the analogs tested, the original pCDR3 is a more efficient modulator of experimental SLE-associated T-cell responses and of disease manifestations. Further, pCDR3 is suggested as a general immunomodulator for the different models of murine lupus considering its beneficial effects on the experimental disease as well as on the spontaneous (NZB × NZW)F1 and MRL lpr/lpr lupus models.

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